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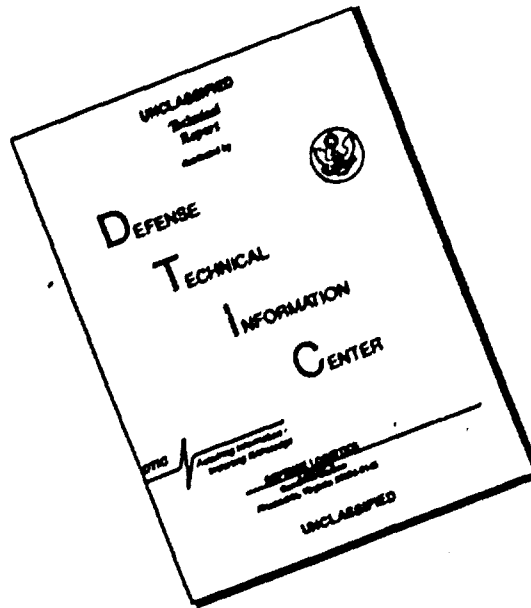
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Increased Efficiency of Retroviral-Mediated Gene Transfer and Expression in Primate Bone Marrow Progenitors After 5-Fluorouracil–Induced Hematopoietic Suppression and Recovery

By Robert Wieder, Kenneth Cornetta, Steven W. Kessler, and W. French Anderson

To define conditions for improved efficiency of retroviral-mediated gene transfer and expression in primate progenitor cells, four rhesus monkeys were treated with a 200 mg/kg intravenous bolus of 5-fluorouracil (5-FU). The kinetics of hematopoietic suppression and recovery were assessed in peripheral blood, bone marrow mononuclear cells, and bone marrow cells fractionated in an albumin density gradient. Bone marrow mononuclear cells were transduced with N2, a retroviral vector carrying the bacterial neomycin phosphotransferase gene (NPT), which confers resistance to the otherwise toxic neomycin analogue, G418. Circulating colony-forming units-granulocyte-macrophage (CFU-GM) disappeared at 2 days. CFU-GM, transducible CFU-GM, CD34$^+$ cells, and the percent of cells in cycle decreased at 3 days in unfractionated bone marrow cells and in a light density population known to be enriched for these progenitors and for stem cells. NPT activity in the light-density fraction,

marginally detectable before treatment, disappeared at 3 days as well. At day 7 the CFU-GM plating efficiency, the CD34$^+$ cell content, and the percentage of cells in cell cycle began to increase in the light-density fraction. The NPT assay became faintly positive again but the CFU-GM were not yet transducible, implying that it was an earlier progenitor population that was dividing and differentiating. By day 15, there was a marked rebound in all of the progenitors measured, and transduction efficiency assessed by G418^R CFU-GM and NPT assay rebounded to several times pretreatment levels. The data suggest that CFU-GM are optimally transduced at 15 days but that earlier progenitors are more likely cycling and transducible before 5 days, a time when a gene transfer experiment would probably have the best chance to succeed.

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RETROVIRAL VECTORS have been used to transfer and express cDNAs in murine[1,2] and human[3] hematopoietic cells in vitro. DNA sequences were also introduced into murine hematopoietic progenitor cells in vivo[4-6] and expression of the transferred gene was obtained after in vitro transduction/syngeneic bone marrow transplantation.[7-9] Long-term in vivo expression in hematopoietic cells showed successful transduction of early, self-renewing hematopoietic progenitors.[10-12] These data have raised the expectation that gene therapy may be useful in treating human genetic disease. An approved clinical trial using retroviral-mediated gene transfer is being performed in humans using the neomycin phosphotransferase (NPT) gene transferred into tumor-infiltrating lymphocytes as a marker gene.[13] Gene transfer into bone marrow progenitors as a therapeutic measure awaits successful completion of primate experiments. To date, long-term retroviral vector expression in primate bone marrow progenitors using amphotropically packaged vectors has met with limited success.[14] Transduction efficiency was low and variable, at best, and periods of expression were of short duration. This suggested that transduction rates of hematopoietic stem cells and early

progenitors were negligible in normal healthy unmanipulated monkeys.

Evidence (primarily in mice) suggests that cell cycling is necessary for retroviral reverse transcription[15-17] and integration.[15,18] Data also suggest the possibility that cell cycling may be necessary for retroviral attachment (in human derived hematopoietic cell lines).[19] One possible reason for the failure of retroviral vectors to transduce primate bone marrow stem cells may be that the latter are in the G_0 phase. We attempted to increase the transduction efficiency of primate bone marrow progenitors by stimulating cell division in vivo with the chemotherapeutic agent 5-fluorouracil (5-FU).

5-FU causes hematopoietic suppression by its cytotoxic effect on dividing bone marrow progenitors.[20] The kinetics of 5-FU–induced hematopoietic suppression and recovery are well documented in mice for a variety of cellular components. Among the populations studied are red blood cells (RBCs),[21] white blood cells (WBCs),[21] neutrophils,[22] B-cell precursors,[23] platelets[21] and megakaryocytes,[24,25] marrow cellularity,[23,26] colony-forming units-granulocyte macrophage (CFU-GM),[21,22,27] colony-forming units-spleen (CFU-S),[21,26,29] megakaryocyte colony-forming units-spleen (MKCFU-S),[25] and high proliferative potential progenitors.[26,27,30] Studies in mice also demonstrated a small, noncycling 5-FU–resistant stem cell population that has the capacity to repopulate the marrow.[28,31,32] Bone marrow recovering from 5-FU insult is enriched for proliferating early progenitors and stem cells, some of which are undergoing self-renewal.[10,11,33] Long-term hematopoietic reconstitution with retrovirally transduced stem cells was often achieved with bone marrow from donor mice pretreated in vivo with 5-FU 2 to 3 days before harvest.[9,11] Pretreatment optimized transfer into a primitive stem cell population.[20,24,29,31,34] Attempts at gene transfer into 5-FU–treated monkeys by using conditions developed for mice have not been successful (Gillio A, O'Reilly R: unpublished results, March 1987; Wieder R, Anderson WF: unpublished results, February 1988). One

explanation for these negative results could be that the kinetics of hematopoietic suppression and recovery after 5-FU insult are different in primates than in mice.

In this report, we investigated the 5-FU-induced suppression and recovery of the rhesus monkey hematopoietic system, including the peripheral and the bone marrow components. We transduced bone marrow samples with N2. a vector carrying the bacterial NPT gene at time points before and after the administration of a 200 mg/kg intravenous (IV) bolus of 5-FU. We assayed the transduction efficiency in vitro in the bone marrow mononuclear cell population and in a light-density fraction shown to be enriched for stem cells (Wagemaker G, personal communication. January 1988), CD34 antigen positive cells, and CFU-C (this study). In mice, this population is preferentially infected by endogenous retroviruses.[35] While recognizing that the only true assay of stem cell transduction is that which measures gene expression after autologous bone marrow transplantation and hematopoietic reconstitution, we measured a number of parameters that provided initial data, indirectly enabling us to make a "best guess" estimate of the optimum time after 5-FU insult to perform these in vivo experiments.

MATERIALS AND METHODS

Primates and hematopoietic specimens. Experiments were performed using four female rhesus monkeys weighing between 4.0 and 4.5 kg who had central venous catheter placed before the experiment (Table 1). The bone marrow studies were performed on three of these animals. Two female rhesus monkeys were used as controls in parallel samplings. Bone marrow aspirations and biopsies were taken from the ischial rami and iliac crests under general ketamine anesthesia using rotating sites such that no site had been aspirated before biopsy, nor had any site been aspirated more than once. The experimental animals each received a single dose of 200 mg/kg 5-FU IV. Peripheral blood samples were drawn from the central venous catheter, initially daily, then 3 to 4 weeks after 5-FU treatment, every 2 to 3 days. Blood and bone marrow samples were aspirated into preservative-free heparin. Peripheral cell counts were done in a clinical hematology laboratory. Biopsy specimens were fixed and stained with hematoxylin and eosin by American Histolabs (Rockville, MD). Blood and bone marrow mononuclear cells were separated using Lymphocyte Separation Medium (LSM; Litton Bionetics, Kensington, MD) and subsequently washed with phosphate-buffered saline (PBS). All cell counts were done manually using exclusion of 0.2% Trypan Blue as the criterion for viability.

Gene transfer and NPT assays. Freshly harvested marrow was transduced using supernatant gene transfer techniques, as previously described.[14] Viral supernatants were harvested from N2/PA317 producer cells[8,14] and frozen. Titers were 2.4 to 6 × 10⁵ G418ᴿ CFU/mL.[8] Transductions were performed with thawed supernatant in the presence of 8 μg/mL polybrene at multiplicities of infection of 3.8 to 5.4 for 2 hours at 37°C. Cells were then pelleted and washed before use in colony assays, NPT assays, or density separations.

Intact mononuclear or density-fractionated transduced cells were incubated for a period of 4 to 5 days in a medium containing 30% heat-inactivated fetal calf serum (FCS), 10% (wt/vol) bovine serum albumin (BSA), 10% lymphocyte-conditioned medium (LCM) of a frequently phlebotomized hemochromatosis patient. 12 mmol/L 2-mercaptoethanol (2-ME), and 2 mmol/L glutamine at 37°C. The cells were then washed and frozen as dry pellets for subsequent NPT assays. NPT assays were performed on cell lysates from 1 to 4 × 10⁶ nucleated cells after separation of NPT from endogenous phosphotransferases using nondenaturing polyacrylamide gel electrophoresis. The transfer of ³²P from [γ³²P]adenosine triphosphate to kanamycin, as previously described,[14] was used to assess NPT activity.

Colony assays. Colony assays were performed as previously described.[14] Briefly, cells were plated in 1-mL vol of 1% methylcellulose solutions containing 30% heat-inactivated FCS, 5% LCM. 10% (wt/vol) BSA, 2 mmol/L glutamine, and 12 mmol/L 2-ME. with and without the presence of 1.2 mg/mL G418 (a concentration shown to be lethal for nontransduced cells). CFU-GM containing an estimated 40 or more cells were counted at 14 ± 2 days.

CD34 antigen determinations. The frequency of the bone marrow cells expressing the CD34 antigen was determined by staining with the anti-CD34 monoclonal IgG2a antibody K6.1.[37] This is a high-avidity antibody that cross-reacts with both human and nonhuman primate CD34 antigen (Kessler SW, manuscript in preparation) similar to the 12.8[38,39] and unlike the MY10[40] monoclonal antibodies (MoAbs) described previously. Secondary staining was performed with phycoerythrin-conjugated goat antimouse κ (Becton Dickinson, Sunnyvale, CA) or with biotin-conjugated goat antimouse Ig (Kirkegaard & Perry Labs, Gaithersburg, MD) antibodies, followed by phycoerythrin-streptavidin (Becton Dickinson) and flow cytometry analyses on the Ortho Cytofluorograf (Ortho Diagnostic Systems, Inc, Westwood, MA). The myeloma protein ADJ-PC5 or MoAb D3-2H2 (anti-Dengue complex; American Type Culture Collection, Rockville, MD) served as isotype-matched negative controls for K6.1.

Albumin density gradients. Density separations of bone marrow mononuclear cells were performed essentially as described by Dicke et al.[41] Mononuclear cells were suspended in 1.5 mL of a final concentration of 17% wt/vol Path-O-Cyte 4 (ICN ImmunoBiologicals, Lisle, IL) BSA solution and layered onto a preformed gradient consisting of four 1.5-mL layers of 25%, 23%, 22%, and 21% wt/vol BSA solutions in a Falcon polystyrene centrifuge tube at room temperature. BSA solutions were prepared by diluting 35% Path-O-Cyte 4 with PBS. Concentrations were determined by

Table 1. Description of Rhesus Monkeys, 5-FU Treatment, and Experimental Sampling Undertaken in the Study

Monkey No	Approximate Age (y)	Approximate Weight (kg)	200 mg/kg 5-FU	Peripheral Counts	Peripheral CFU-C	Marrow Aspiration	Marrow Biopsy
055E	4	4.2	+	+	−	−	
079E	4	4.5	+	+	+	+	
0338	4	4 0	+	+	+	+	+
102E	4	4.0	+	+	+	+	+
045	8	7.5	−	−	+	+	
89789	8	8	−	+	+	+	

All animals were females

refractive index measurements. Gradients were centrifuged at 1,000g for 30 minutes at 20°C in a Sorvall RC3B centrifuge. The cells segregated at the interfaces formed by the solutions and at the bottom of the tube. The interfaces and the pellet were labeled layers 1 through 5 in descending order. After centrifugation, the cells were removed sequentially from the top with a 1-mL pipette.

Cell cycle analysis. Cells were analyzed for DNA content on an Ortho Cytofluorograf after staining with propidium iodide using a procedure described by Krishan.[42] The distribution of cells within the cell cycle was determined by the method of Dean and Jett[43] using a program written by Dr P.S. Rabinovitch (University of Washington, Seattle).

RESULTS

Peripheral cellularity. Administration of a 200 mg/kg IV bolus of 5-FU to four animals affected the concentration of all circulating blood cell lineages. The hematocrit decreased and recovered after a nadir 5 to 9 days after 5-FU administration (Fig 1A). The reticulocyte count began to increase between 5 and 10 days after 5-FU insult (Fig 1B). Two of the animals were transfused at 5 and 7 days (animal 0338) and at 8 days (animal 079E) because of medical indications. The WBC and absolute neutrophil counts also decreased and then recovered after a nadir 11 to 15 days after the 5-FU bolus (Fig 1, C and D). Platelet counts were not significantly depressed but a thrombocytosis was observed between 16 and 18 days after 5-FU insult (Fig 1E).

Peripheral colony progenitors. The plating efficiency of circulating mononuclear cells (CFU-GM/10⁶ cells) declined to zero 2 days after 5-FU administration (Fig 1F). It began to recover 13 to 15 days after treatment, and rebounded, in some instances, to 10 times the pretreatment plating efficiency before returning to starting levels at 25 to 30 days.

Bone marrow cellularity. Bone marrow biopsies performed on two animals showed a decrease in cellularity 3 days and 7 days after 5-FU treatment and a return to greater than pretreatment cellularity on day 15. Day 7 biopsies showed an increase in erythroid progenitors. The average cellularity of interval bone marrow aspirates from three 5-FU–treated monkeys is outlined in Table 2. The number of mononuclear cells per mililiter of aspirate decreased by two thirds on day 3, slightly increased on day 7, and recovered to above baseline at 15 days. The cellularity rebounded to 150% of pretreatment levels at 24 days.

Bone marrow progenitor populations and cell cycling status. Mononuclear cells bearing the CD34 surface antigen (CD34⁺), CFU-GM, and CFU-GM transduced by N2 were all affected by 5-FU treatment (Table 2). The percentage of CD34⁺ cells decreased to 32% of pretreatment value at 3 days, then increased to approximately 43%, 73%, and 53% of pretreatment value at 7, 15, and 24 days, respectively. The mononuclear cell CFU-GM plating efficiency decreased to approximately 15% of baseline at 3 days, remained unchanged at 7 days, and returned to pretreatment value at 15 days. The G418[R] CFU-GM plating efficiency declined to near zero at 3 days, remained absent at 7 days, and rebounded to 26%, nearly fivefold the pretreatment value, at 15 days, before returning to baseline at 24 days. Fluorescent-activated cell sorting by DNA content was used to determine the cell cycle status of the intact mononuclear population. The fraction of cells undergoing cell division decreased by one half at 3 days, to 7.2%, then increased progressively to 8.3% at 7 days, 11.2% at 15 days, and 13.7% at 24 days after 5-FU insult (Table 2).

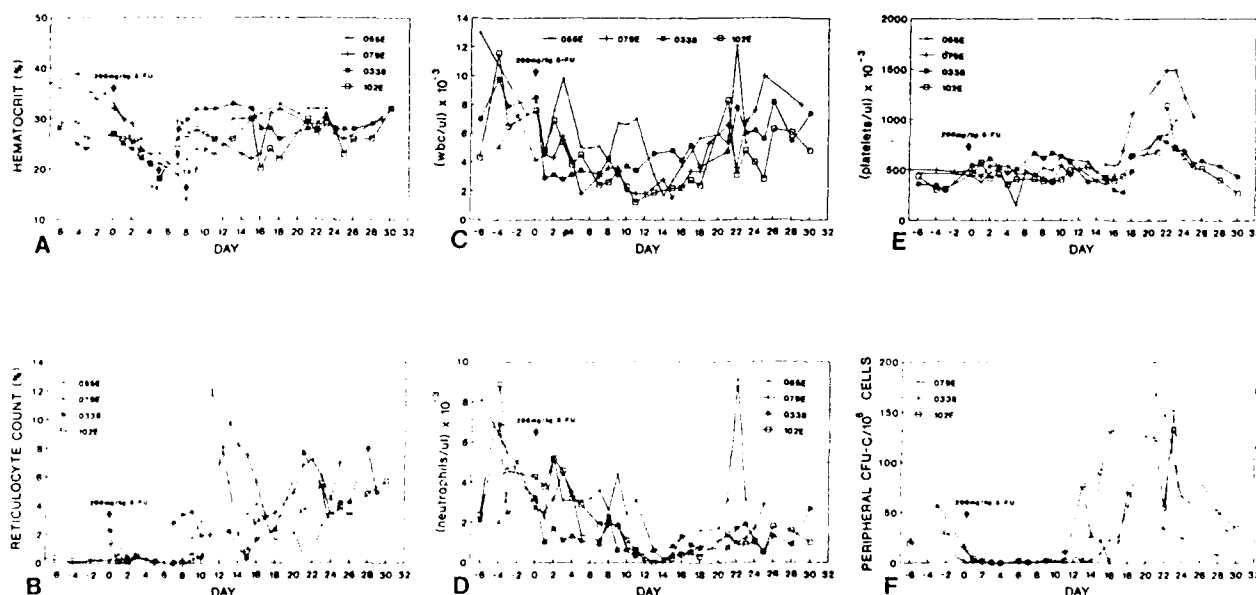The range of values reported in Table 2 is quite broad for

Fig 1. Quantitative changes in peripheral hematopoietic elements in four rhesus monkeys (055E, 079E, 0338, and 102E), each administered a 200 mg/kg IV bolus of 5-FU. The hematopoietic elements assayed were: (A) hematocrit; (B) reticulocyte count; (C) WBC count; (D) absolute neutrophil count; (E) platelet count; and (F) peripheral CFU-GM (not assayed in 055E). Tx with an arrow indicates transfusion of animal 0338 at 5 and 7 days and of 079E at 8 days.

Table 2. Cellularity, Progenitor Frequency, Transduction Efficiency, and Cycling Status of the Intact Bone Marrow Mononuclear Population as a Function of Time After 5-FU Treatment

	Day 0	Day 3	Day 7	Day 15	Day 24
Cells/mL $(\times 10^{-6})$	15	4.7	6.2	17	23
	(12-19)*	(4.3-5.1)	(3.6-10.5)	(8-21)	(17-31)
% CD34⁺ cells	24.8	7.9	10.6	18.1	13.2
	(5.7-41.5)	(4.7-13.4)	(8.5-13.3)	(2.0-36.9)	(8.2-18)
CFU-GM/10^5 cells	37.6	5.75	5.7	38.4	39.0
	(28.8-42.8)	(2.5-8.0)	(3.5-7.0)	(2.2-84.4)	(28.8-57.3)
G418ᴿ CFU-GM/10^5 cells	2.2	0.08	0	10.0	1.1
	(0.25-4.0)	(0-0.25)		(0.25-22.5)	(0.75-1.5)
% Transduction†	5.9	1.4	0	26.0	2.8
	(0.6-9.3)	(0-3.1)		(11.4-26.5)	(1.7-4.8)
% Cells not in G1/G₀	15.3	7.2	8.3	11.2	13.7
	(9.0-27.3)	(4.9-8.6)	(1.7-16.9)	(5.1-16.9)	(11.5-20.1)

*Numbers in parentheses are the range of mean values in the three animals.

†Mean (G418ᴿ CFU-GM/10^5 cells/mean CFU-GM/10^5 cells) × 100% (ranges represent mean transduction efficiencies for each animal assayed).

all parameters assayed. The animals exhibited considerable biologic variability with respect to bone marrow response to the 5-FU insult. One animal in particular was affected far more than the others, causing her recovery to lag behind as well. However, the trends and behavior of the subpopulations studied were analogous in all of the animals studied.

Albumin density gradient fractionation. Progenitor populations and cell cycling were measured in density-separated bone marrow cell populations fractionated in an albumin step gradient. The CD34⁺ cell content of each layer is shown in Fig 2. The top three layers had a higher CD34⁺ cell content at baseline than did unfractionated mononuclear cells. The frequency of CD34⁺ cells decreased throughout the gradient at 3 days after treatment. It rebounded in the top layer at 7 days and reached nearly 70% at 15 days, markedly shifting its distribution to favor the top of the gradient. At 24 days, the distribution returned to the

Fig 2. Quantitative changes in the percentage of CD34+ bone marrow cells in each layer of an albumin density step gradient as a function of time after a 200-mg/kg IV bolus of 5-FU in three rhesus monkeys: 079E, 0338, and 102E (mean). Layers 1, 2, 3, 4, and 5 represent the interfaces of the 17% to 21%, 21% to 22%, 22% to 23%, 23% to 25% solutions and the bottom of the gradient, respectively.

pattern at baseline, although average values decreased to below the starting levels.

The average numbers of CFU-GM/10^5 cells and G418ᴿ CFU-GM/10^5 cells were highest in the upper layers of the gradient at baseline (Table 3). The plating efficiency decreased throughout the gradient at 3 days, but to a greater degree in the lower layers. A partial recovery was seen in the upper layers on day 7, and a universal rebound was observed at 15 and 24 days. At 3 and 7 days after treatment, the number of G418ᴿ CFU-GM/10^5 cells decreased to zero in all but the top layer. There was a marked rebound in transduction rates in all of the layers at 15 days, reaching about 30% in the top three layers. By day 24, the transduction rates withdrew to approximately pretreatment levels. Standard error for each animal averaged about 30% but was often higher than 70% when there were only one or two colonies per plate. A control animal's fractionated bone marrow did not show a trend in CFU-GM, and the percent G418ᴿ CFU-GM fluctuated between 0 and 5% in unfractionated marrow.

The changes in the distribution of mononuclear cells, of the absolute number of CD34⁺ cells, of CFU-GM, and of G418ᴿ CFU-GM are shown in Fig 3. At baseline, most of the cells distributed in the bottom two layers of the gradient. The CD34⁺ cells, although enriched in the top fractions, still distributed mostly in the bottom two layers. The CFU-GM and G418ᴿ CFU-GM had a bimodal distribution. At 3 and 7 days after 5-FU administration there was a moderate shift toward the upper layers in the mononuclear cells and the CD34⁺ cells, and a marked shift in the CFU-GM and G418ᴿ CFU-GM. By day 15 there was a remarkable recovery in all of the cellular and progenitor elements in the lower layers as the marrow was reconstituting and differentiating. This pattern remained similar at 24 days.

NPT assay. NPT expression after N2 transduction was not detected in unfractionated marrow before 5-FU treatment, nor in the control animals. Faint bands in layer one and occasionally layer two of fractionated marrow before treatment disappeared at 3 days and reappeared at 7 days
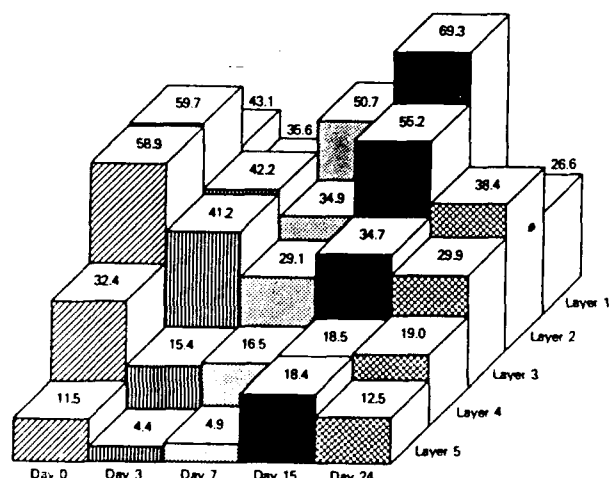
Table 3. Frequency of CFU-GM and G418^R CFU-GM in N2 Supernatant-Transduced/Density Fractionated Mononuclear Cells as a Function of Time After 5-FU Treatment

Day	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
CFU-GM/10^5 cells					
0	135.5	99.1	49.0	28.0	15.0
	(30-208.5)*	(94.5-102.8)	(33.9-76.3)	(15.9-44.3)	(3.0-21.7) .
3	64.6	13.3	2.25	0.75	0
	(30.9 86.4)	(1.5-36.0)	(0-3.75)	(0-1.5)	
7	94.5	28.4	5.5	1.75	1.25
	(72.0-145.0)	(2.3-62.4)	(3.0-9.0)	(0-3.0)	(0-3.0)
15	202.3	214.0	71.6	38.3	24.0
	(40.5-342.9)	(21-421.5)	(12.9-125.4)	(5.4-89.4)	(3-48.0)
24	275.3	220.5	121.0	59.0	31.3
	(159-452.5)	(102.9-284.1)	(78-150.8)	(10.5-84.8)	(6.75-5.1)
G418^R CFU-GM/10^5 cells [% transduction]†					
0	12.0 [8.8]	6.1 [6.2]	2.3 [4.6]	0.2 [0.6]	0.8 [5.2]
	(1.5-21.0)	(0-16.0)	(0-6.75)	(0-0.5)	(0-2.4)
3	0.8 [1.2]	0.1 [0.6]	0 [0]	0 [0]	0 [0]
	(0-1.5)	(0-0.75)			
7	1.0 [1.1]	0 [0]	0 [0]	0 [0]	0 [0]
	(0-1.5)				
15	54.3 [26.8]	63.8 [29.8]	18.3 [25.6]	5.0 [10.4]	3.5 [14.6]
	(9.75-117.0)	(1.5-177.0)	(1.5-42.9)	(0-13.5)	(0-9.0)
24	15.3 [5.5]	13.2 [6.0]	5.0 [4.1]	2.67 [4.5]	1.0 [4.1]
	(4.5-26.3)	(4.5-20)	(0-13.5)	(0-6.0)	(0-1.5)

*Numbers in parentheses are the range of mean values in the three animals.

†G418^R CFU-GM/10^5 cells/CFU-GM/10^5 cells.

post 5-FU administration. On day 15 and 16, there was markedly increased NPT activity in the top three layers, and on day 24 in the top four layers. On day 24, intact marrow turned positive, and the NPT band became more intense in the second layer than in the first layer.

Effects of 5-FU on cell cycling. Throughout the period of marrow suppression and recovery, the fraction of cells undergoing cell cycling was greater in the upper layers of the gradient (Table 4). Three days after 5-FU treatment, the cycling fraction decreased universally. On day 7, the cells in most layers increased their cycling fraction. A continued increase was measured on day 15, with cells in the bottom layers recovering to pretreatment levels. The more primitive less dense cell populations only recovered to approximately 75% of their pretreatment cycling fractions and remained at that level at 24 days.

DISCUSSION

Based on in vitro data suggesting that cell cycling is necessary for completion of the steps from retroviral attachment to expression of the integrated transduced gene,[15,19] we cycled primate bone marrow in vivo, then transduced samples in vitro with a retroviral vector carrying the bacterial NPT gene. Our aim was to define the post 5-FU-insult time curve of transduction efficiency assayed by NPT activity and G418^R CFU-GM in a light-density bone marrow cell subpopulation partly enriched for early progenitors. We tried to correlate the transduction efficiency with the hematopoietic recovery and cell cycle status. While recognizing the difficulty of making inferences about stem cells using data obtained from a more heterogeneous population, we tried to obtain a "best guess" estimate of

when the earliest progenitors would be cycling. During such a time, the early progenitors, according to the hypothesis, would be susceptible to retroviral transduction and durable gene transfer.

We defined the kinetics of hematopoietic suppression and recovery by monitoring cell populations at different stages of differentiation in the marrow and in the circulation. The first cells in which we documented an effect were the circulating CFU-GM, a population of relatively late progenitors that disappeared after 2 days.[44] At 3 days we showed a decrease in the bone marrow CD34$^+$ cell population, and also in CFU-GM and transducible CFU-GM. These cells were destroyed by 5-FU as the majority, most likely, were undergoing cell division.

A greater diminution was observed in the cellularity, the CFU-GM, and the transducible CFU-GM content of denser layers at 3 days. This effect was probably caused by the maturation of cells in those compartments and their entrance into the circulation. The upper-layer progenitors that would normally differentiate to denser cells were destroyed by 5-FU, resulting in the net depletion of the cells distributing in the lower layers. The circulating blood cells decreased as a result of phlebotomy (RBCs), senescence, and nonreplacement by depleted marrow precursors on a more protracted time course.

The sequential steps of hematopoiesis were reflected in the recovery. Between days 5 and 10, the reticulocyte count and the hematocrit began to increase. The bone marrow biopsy reflected this on day 7 when a predominance of erythroid precursors was present. The CD34$^+$ cells and the CFU-GM began to increase in the top layer on day 7, indicating the beginning of early differentiation. This was
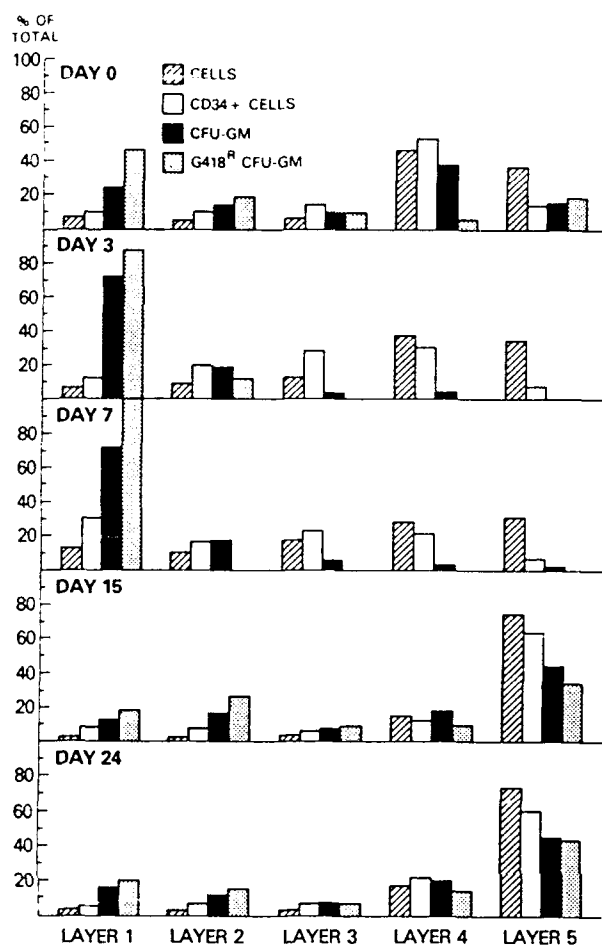
Fig 3. Quantitative changes in the distribution of N2 supernatant-transduced bone marrow mononuclear cells, CD34 antigen positive cells, CFU-GM, and G418[R] CFU-GM in an albumin density gradient as a function of time after a 200-mg/kg IV bolus of 5-FU. Layers 1, 2, 3, 4, and 5 represent the interfaces of the 17% to 21%, 21% to 22%, 22% to 23%, 23% to 25% solutions and the bottom of the gradient, respectively. The values for cells represent the normalized percentage of mononuclear cells in a layer. The other values are the arithmetic product of the percentage of mononuclear cells in a layer (from this figure) and (a) the percent CD34+ cells (from Fig 2), or (b) the number of CFU-GM/10⁵ cells, or (c) of G418[R] CFU-GM/10⁵ cells in that layer (from Table 3), normalized over the five layers.

roughly 1.5 times that of intact marrow. The NPT assay showed a very faint band. Layer 1 contained 6% of the cells at baseline, but over 20% of the CFU-GM and almost 50% of the G418[R] CFU-GM (Fig 3). Approximately 34% of the cells were undergoing cell cycling (Table 4) compared with 15% of the unfractionated mononuclear cells. The percentage of the cells positive for the CD34 antigen was 43% compared with 24% for the intact population.

At day 3, the cycling cells in layer 1 were at 36% of the pretreatment level (Table 4), the CFU-GM plating efficiency was at 50% of the pretreatment level (Table 3), and the CD34⁺ cells were at 72% of the starting levels (Figure 2). The CFU-GM that were present were not transducible, and the NPT assay of all cells in layer 1 was negative as well. The fact that the CFU-GM were more diminished than the whole CD34⁺ cell population suggests that the CD34⁺ cells represented a more primitive population at this point. The nontransducibility of CFU-GM suggests that these relatively differentiated CD34⁺ cells are not cycling. However, it cannot be ascertained from the data whether the cycling cells represented earlier CD34⁺ cells or cells that were CD34 negative, which have differentiated beyond the CFU-GM stage and were no longer clonable.

On day 7, the frequency of CFU-GM in layer 1 had increased by 50% over their frequency on day 3, but the CFU-GM were still not transducible. The percentage of CD34⁺ cells had increased to above pretreatment levels, however, and the percentage of cells undergoing cell division increased from 12% to 17%.

The increase in CFU-GM and CD34⁺ cells represented division and differentiation of earlier progenitors. The disparity in the rate of recovery of CD34⁺ cells (to 118% of baseline) and CFU-GM (to 70% of baseline) in layer 1 on day 7 suggests that most of the recovery was in the pre–CFU-GM CD34⁺ cell population. The plating efficiency of more differentiated CD34⁺ cells (cells that are CD33⁺ as well) is 7 to 10 times that of earlier CD34⁺ cells (cells that are CD33⁻).[46] The fact that the CFU-GM are not transducible suggests that this new population of CFU-GM are not yet undergoing cell division.

Thus, the increase in the percent of cycling cells probably reflects the pre–CFU-GM CD34⁺ population. The shift in cellularity toward the top layer further supports the suggestion that maturation had not yet progressed beyond the CFU-GM point of differentiation. The NPT assay again became weakly positive, probably representing this pre–CFU-GM CD34⁺ population, which was undergoing cell division. Because 50% of the cells in layer 1 were not CD34⁺, it is feasible, albeit unlikely, that other, more differentiated cells were being transduced and contributed to the positive NPT assay. This is disputed by the discussion above.

At day 15, the CFU-GM plating efficiency had rebounded to 150% of pretreatment levels and the G418[R] CFU-GM to 300% of pretreatment levels. The NPT assay was markedly positive, and the percentage of cells undergoing cell division was up to 75% of baseline levels. The cycling cells were probably at the stage of differentiation of CFU-GM, reflecting these increased plating and transduc-

supported by the increase in the percentage of cells undergoing cell division on day 7 in the upper layers. The percentage of cycling cells continued to increase throughout recovery, but remained below the pretreatment value. The bone marrow cells were more synchronized than at baseline; however, and cycling cells probably represented less differentiated cell populations than before treatment. By day 15, the distribution of cells, CD34⁺ cells, CFU-GM, and G418[R] CFU-GM changed to the more differentiated lower layers of the gradient, and the circulating CFU-GM and WBCs all began to recover. The CFU-GM kinetics are similar to those found by others.[45]

Transduction results may best be analyzed by discussing data from cells in layer one of the gradient. Baseline CFU-GM transduction efficiency in layer 1 was about 9%,

Table 4. Percentage of Density Fractionated Cells not in the G1/G0 Phase of the Cell Cycle as a Function of Time After 5-FU Treatment

Day	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
0	34.3	20.6	18.2	9.9	11.7
	(30.1-37.7)*	(14.7-31.4)	(9.1-36.1)	(7.7-13.7)	(8.4-13.7)
3	12.5	9.9	3.8	4.9	7.6
	(4.9-22.6)	(8.9-11.6)	(3.2-4.3)	(3.5-6.1)	(6.7-8.6)
7	17.0	12.6	7.1	5.0	9.9
	(9.4-21.8)	(7.9-18.3)	(3.7-10.7)	(1.7-8.4)	(1.5-19.1)
15	25.1	16.0	13.4	10.2	11.3
	(19.5-30.3)	(8.0-23.9)	(4.2-20.2)	(5.8-15.8)	(6.7-17.2)
24	24.7	16.9	12.1	8.2	13.6
	(22.2-28.6)	(16.4-17.2)	(10.9-14.2)	(4.9-11.7)	(11.2-17.7)

*Numbers in parentheses are the range of values in the three animals.

tion efficiencies. The cellularity of layer 1 dropped to 40% of pretreatment levels (Fig 3) because the majority of the marrow cells were more differentiated and segregated in the bottom layers. The CD34⁺ cells also increased to 160% pretreatment levels, representing an expansion of the later progenitor pool consisting in part of the CFU-GM.[47]

The best time to transduce CFU-GM, based on our sampling times, was 15 days after 5-FU insult. At day 7, the evidence suggests that the cells in layer 1 were probably of an earlier lineage than CFU-GM and that the increase in cycling from day 3 to day 7 was representative of the differentiation of earlier progenitors into CFU-GM. There is further suggestion that the earlier progenitors may have already been undergoing cell division before day 5 when reticulocytes began to appear in the circulation of some of the animals. We have no data with respect to early progenitor behavior at day 3. One could speculate that the earliest progenitors would be susceptible to transduction by a retroviral vector sometime before day 7 and possibly before day 5 after 5-FU insult, assuming they behaved as CFU-GM with respect to transduction efficiency and cycling.

ACKNOWLEDGMENT

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